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NOTES

Antibody Response of Humans to the Circumsporozoite Protein of *Plasmodium vivax*

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We studied the interaction of sera from residents of an area in northern Peru where vivax malaria is endemic with four recombinant DNA-derived circumsporozoite (CS) proteins of *Plasmodium vivax*. The antigens used in the enzyme-linked immunosorbent assay included one *Escherichia coli*-produced and three *Saccharomyces cerevisiae*-produced recombinant proteins. Three of the proteins ($NS1_{81}V20$, Vivax-1, and Vivax-2) contain the entire central repeat region of the *P. vivax* CS protein, and one protein (Vivax-3) contains only two repeat sequences. Vivax-1, Vivax-2, and Vivax-3 contain different lengths of sequences flanking the repeats. A higher percentage of the sera had antibodies to Vivax-2 and Vivax-3, the two proteins containing the longest nonrepeat sequences, than to $NS1_{81}V20$ or Vivax-1. Children less than 5 years of age did not have immunoglobulin G antibodies to $NS1_{81}V20$; however, they had antibodies to Vivax-1, Vivax-2, and Vivax-3. The finding that individuals living in a malaria-endemic area produce antibodies to peptides containing nonrepeat regions of the CS protein emphasizes the need to characterize the immune response to these regions in naturally exposed and experimentally immunized humans.

The circumsporozoite (CS) protein of *Plasmodium vivax* has been identified as a target antigen for a human malaria vaccine. The native CS protein of *P. vivax* is 373 amino acids in length and contains a central region of tandem repeats of nine amino acids. Sequences designated regions I and II flank the repeats and are nonhomologous among many species of malaria (1–4).

We studied the interaction of serum samples from individuals residing in a malaria-endemic area in northern Peru with four recombinant DNA-derived CS proteins of *P. vivax*. These four proteins contain different lengths of the CS molecule. The recombinant protein $NS1_{81}V20$ (SmithKline and French, Swedeland, Pa.) consists of 81 amino acids from the nonstructural protein 1 of influenza A virus fused N-terminally to the entire central repeat region of the CS protein of *P. vivax*. The Vivax-1, Vivax-2, and Vivax-3 proteins (Chiron Corporation, Emeryville, Calif.) are recombinant products expressed internally in *Saccharomyces cerevisiae* (1, 2). Vivax-1 contains the entire repeat region plus 15 amino acids preceding the repeats and 48 amino acids flanking the repeats at the carboxy-terminal end. Vivax-2 differs from Vivax-1 by containing an additional 25 amino acids at the carboxy-terminal end (1). Vivax-3 differs from Vivax-2 in that it contains an additional 54 amino acids at the N-terminal end and only one of each of the repeat sequences DRADGQPAG and DRAVGQPAG (2).

The study population consisted of 311 volunteers, ranging in age from 1 to 88 years, who resided in one of five villages (Andoas Viejo, Andoas Nuevo, Alan Garcia, Libyacu, and La Bandat near the Occidental Petroleum base camp in Andoas, Peru. Malaria caused by *P. vivax* is endemic in this area. There were no reports of *P. falciparum* infections in residents during the 6 years that records were kept. The prevalence of malaria was highest in children between the ages of 1 and 9 (17% slide positive) and lowest in adults (>16 years; 7% slide positive). All infections were caused by *P. vivax*. Immunoglobulin G (IgG) antibody titers to blood stage *P. vivax* were measured by an indirect immunofluorescence assay. The proportion with positive titers (>1:32) increased with age; 36% of those 1 to 9 years old and 54% of those >16 years old had antibodies.

The presence of specific IgG antibodies against the recombinant CS proteins was determined by an enzyme-linked immunosorbent assay (ELISA) procedure modified from that of Wirtz et al. (11). Briefly, each serum sample was tested in triplicate at a 1:100 dilution in the wells of a 96-well plate (Immulon-2; Dynatech, Chantilly, Va.) coated with either $NS1_{81}V20$, Vivax-1, Vivax-2, or Vivax-3 at a concentration of 2 μg/ml and in triplicate wells without antigen. When testing for antibodies to Vivax-1, Vivax-2, and Vivax-3, 1% yeast extract (Difco, Detroit, Mich.) was added to the blocking buffer and serum diluent. The optical densities (ODs) were read at 410 nm after 60 min. The mean difference between the OD of triplicate serum samples in wells with and without antigen was reported as the OD. A positive antibody response was one in which the OD of the test serum was greater than the mean OD plus 3 standard deviations for serum samples from 20 individuals with no prior exposure to *P. vivax*. Positive ODs for $NS1_{81}V20$, Vivax-1, Vivax-2, and Vivax-3 were >0.065, 0.035, 0.045, and 0.085, respectively.

The percentage of individuals with IgG antibodies to each of the four recombinant CS proteins by age groups is shown in Table 1. The >16-year-old group had the highest prev-

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TABLE 1. Positive IgG antibody responses to four antigens representing the *P. vivax* CS protein

Age (yr)	No. tested	% Positive responses			
		$NS1_{81}V20$	Vivax-1	Vivax-2	Vivax-3
1–4	44	0	9	14	21
5–9	73	16	12	12	20
10–15	63	10	10	19	25
≥16	131	24	22	27	19
Total	311	16	15	20	21

lence of IgG antibodies to all proteins except Vivax-3. None of the children 4 years of age and younger had IgG antibodies to $NS1_{81}V20$, whereas they did have antibodies to Vivax-1, Vivax-2, and Vivax-3. Overall, a higher percentage of the serum samples had antibodies to Vivax-2 and Vivax-3 than to $NS1_{81}V20$ or Vivax-1. Only 3% of the samples had antibodies to all four proteins. Four percent of the samples had antibodies to the three proteins containing the complete repeat region but did not have antibodies to Vivax-3. Sixty percent of the samples with antibodies to Vivax-3 also had antibodies to at least one of the other three proteins.

To determine the specificity of the antibody reactions, selected serum samples were tested in an inhibition ELISA. Serum samples diluted 1:100 were incubated with 40 μg of either Vivax-1, Vivax-3, or $NS1_{81}V20$ per ml or with blocking buffer for 2 h at 37°C. Vivax-2 was not tested in this assay. Antibody levels to each recombinant CS protein were determined in the preincubated serum samples with an ELISA. The percentage of inhibition of binding by each protein was calculated as the mean OD of serum samples preincubated with a protein divided by the mean OD of triplicate serum samples preincubated with blocking buffer, multiplied by 100. The results of these assays are shown in Table 2. Inhibition was considered significant when it was 45% or greater. Homologous proteins inhibited binding 82 to 100%.

TABLE 2. Inhibition of binding of serum IgG antibodies to homologous and heterologous antigen

Antibodies in serum sample	Serum no.	Antigen used in ELISA	% Inhibition by		
			Vivax-2	Vivax-3	$NS1_{81}V20$
Vivax-3 only	HEP1036	Vivax-3	0	100	3
	HEP1057	Vivax-3	23	94	0
Vivax-1, -2, and -3 only)	HEP1062	Vivax-1	99	99	5
		Vivax-3	97	97	1
	HEP1066	Vivax-1	95	77	12
		Vivax-3	93	96	11
	HEP1383	Vivax-1	82	56	39
		Vivax-3	0	96	0
$NS1_{81}V20$ and Vivax-1, -2, and -3	HEP1077	Vivax-1	99	10	98
		Vivax-3	29	100	37
		$NS1_{81}V20$	100	14	100
	HEP1104	Vivax-1	86	0	93
		Vivax-3	68	96	0
		$NS1_{81}V20$	95	0	0
	HEP1417	Vivax-1	99	0	89
		Vivax-3	89	84	84
		$NS1_{81}V20$	91	0	83

The reactivity of two serum samples with antibodies only to Vivax-3 was inhibited by preincubation with the homologous CS protein but not with heterologous protein. The reactivity of serum samples positive for Vivax-1 and Vivax-3 was inhibited by preincubation of these sera with either protein but not with $NS1_{81}V20$. In serum samples positive for all four proteins, binding to $NS1_{81}V20$ or Vivax-1 was inhibited by preincubation with $NS1_{81}V20$ or Vivax-1 but not with Vivax-3; binding to Vivax-3 was inhibited by preincubation with Vivax-3 and, for two serum samples to a lesser extent, by Vivax-1. The observation that $NS1_{81}V20$ and, for some sera, Vivax-1 are unable to inhibit binding to Vivax-3 suggests that these antibodies are directed against epitopes outside the repeats that are not present in $NS1_{81}V20$ or Vivax-1. Nonrepeat regions are absent from $NS1_{81}V20$, and Vivax-1 contains a shorter nonrepeat sequence than Vivax-3. It is unlikely that antibodies were directed against a contaminant present in Vivax-1 or Vivax-3, because serum samples from individuals with no exposure to malaria did not have antibodies to these proteins.

The results of the present study indicate that individuals living in a malaria-endemic area produce antibodies to recombinant proteins containing the repeat and nonrepeat regions of the CS protein of *P. vivax*. Data presented here suggest that at least some of these antibodies were directed against epitopes in the nonrepeat region of the CS protein. The percentage of individuals with IgG antibodies to Vivax-2 and Vivax-3, the two recombinant CS proteins containing the longest nonrepeat sequences, was greater than the percentage with antibodies to the protein containing only the repeats ($NS1_{81}V20$) or the protein containing the shortest nonrepeat sequence (Vivax-1). Children below the age of 5 years did not have IgG antibodies to $NS1_{81}V20$; however, 9, 14, and 21% of these children had IgG antibodies to Vivax-1, Vivax-2, and Vivax-3, respectively. The low prevalence of antibodies to $NS1_{81}V20$ could be explained by the possible presence in our study area of variant CS repeats, such as that reported by Rosenberg et al. (8) for *P. vivax* from Thailand. It is unlikely that the antibodies measured in the ELISA were directed against a yeast contaminant in Vivax-1, Vivax-2, and Vivax-3, because serum samples obtained from individuals with no history of malaria infection did not have antibodies to the yeast-derived proteins. Furthermore, in serum samples with antibodies to all four recombinant proteins, Vivax-3 was unable to inhibit binding to Vivax-1 or $NS1_{81}V20$ (Vivax-2 was not tested), suggesting the presence of multiple antigenic sites in the repeat region and outside the repeats in the sequences that are absent from $NS1_{81}V20$ and Vivax-1. It is possible that conformational differences exist among the four recombinant proteins and that epitopes are optimally exposed in proteins containing longer nonrepeat sequences.

Previous studies have demonstrated that the nonrepeat regions of the CS protein of *P. knowlesi* are immunogenic in experimentally immunized monkeys (9) and rabbits (10). Recently it was shown that some individuals in malaria-endemic areas produce antibodies to region I (7). T-cell epitopes in the vicinity of region II have been identified by George et al. (3), who used mice immunized with the peptide PV-23, and by Nardin et al. (5), who used chimpanzees immunized by multiple exposures to the bites of *P. vivax*-infected mosquitoes. Region II was recently shown to con-

tain an amino acid sequence that promotes the adhesion of a variety of human hematopoietic cell lines (6).

In order to precisely define the epitopes to which the antibodies in the Peruvian serum samples are directed, it will be necessary to determine responses to a series of peptides containing overlapping sequences covering the repeat and nonrepeat regions. The results of the present study emphasize the need to further characterize the immune response to the nonrepeat regions in naturally exposed and experimentally immunized humans.

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